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Immunochemical Detection of a Common Antigen Among *Streptococcus uberis* Isolates

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Fifty-three isolates of *Streptococcus uberis* from various sources were examined for the presence of a common antigen. Initially, a serum was produced in rabbits which, by using rocket line immunoelectrophoresis, proved to react with identity to all of the *S. uberis* crude extracts as well as group B and E streptococcal extracts. The antigen(s) responsible for this cross-reactivity was partially purified by Sephacryl S-200 gel chromatography and analyzed by fused rocket immunoelectrophoresis. Further analysis by immunodiffusion showed that probably two antigens in the gel chromatography-consolidated fractions were common to the *S. uberis* and group B and E isolates, but that one of the antigens present was unique to *S. uberis*. Trypsin destroyed the immunoreactivity of this antigen. Isolation of this common antigen could possibly alleviate some of the tedium associated with the identification of this organism.

Streptococcus uberis, since the time of its initial isolation from a case of bovine mastitis in 1922 (1), has remained serologically obscure. In fact, the only reliable method of identifying this organism is through a battery of time-consuming biochemical tests. Most of the serological, as well as ecological, epidemiological, and biochemical, aspects of *S. uberis* have been covered extensively in a review by Cullen (6). Serotyping of *S. uberis* resulted, for the most part, in the establishment of 3 to 11 different types. The serological grouping of *S. uberis* with Lancefield grouping sera has primarily resulted in approximately 20% of these organisms reacting with group E-specific serum. Occasional reactions, however, have been reported with groups B, G, P, and U (7, 14–18). No common antigen has been found for this organism, although Cullen (6) isolated an antigen which was present in his group E-positive isolates. In only one instance was a serum produced against an *S. uberis* strain which would react with all of the other *S. uberis* strains used in that study (22), but the study was of limited scope with regard to *S. uberis* (eight strains total).

With this serological uncertainty in mind, this study was undertaken to determine whether a common, or group, antigen could be detected from a representative sampling of *S. uberis* isolates.

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MATERIALS AND METHODS

Bacterial strains. Fifty-three isolates of *S. uberis* were obtained from various sources: 34 from New York State Mastitis Control Laboratories and the New York State College of Veterinary Medicine, 18 through the courtesy of E. I. Garvie of the National Institute for Research in Dairying in England, and 1 from the American Type Culture Collection (ATCC 19436).

The group B streptococcal strains of various types (Ia, Ib, Ic, II, and nontypable) were obtained from the collection of this laboratory. Group E streptococci were obtained through the generosity of G. E. Wessman, National Animal Disease Laboratory, U.S. Department of Agriculture, Ames, Iowa, and the ATCC (12390 and 21223).

Differentiation of the *S. uberis* isolate from composite milk samples was accomplished by using a battery of biochemical tests as prescribed by McDonald and McDonald (12) and the National Mastitis Council (13).

Crude antigen extraction. Cell wall antigens of the streptococcal strains were extracted according to the method of Baker et al. (3). A 5-ml amount of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) modified with dibasic sodium phosphate (2.8 g/liter) and D-glucose (14 g/liter) (2) was inoculated with the desired organism, incubated for 18 to 24 h at 37°C, and subsequently inoculated into 5 liters of the modified Todd-Hewitt broth and incubated for an additional 18 to 24 h at 37°C. Cultures were centrifuged at 20,000 × *g* for 15 min, washed twice in sterile 0.85% saline, and resuspended in 50 ml of the extraction buffer (0.05 M Na_2HPO_4, 0.15 M NaCl, 0.01 M disodium EDTA, pH 7.4). Glass beads (4-mm diameter) were added to the suspension, which was shaken overnight at room temperature and then centrifuged at 48,300 × *g* for 20 min. The resulting supernatant was filtered through a 0.22-μm membrane filter (Nalge, Rochester, N.Y.)

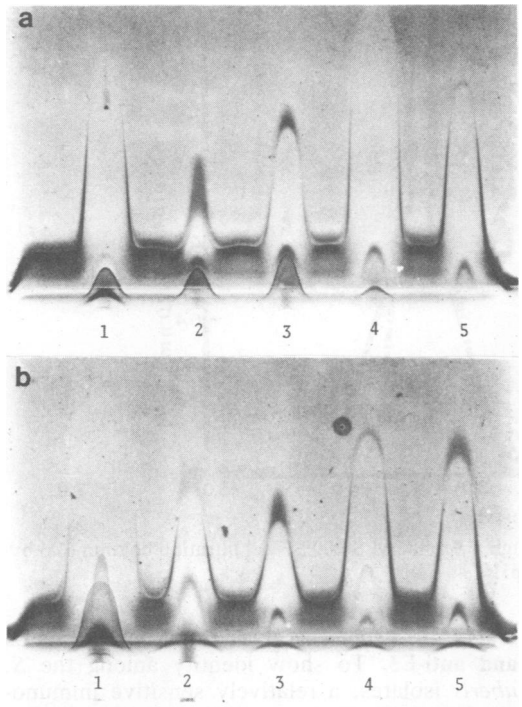

FIG. 1. RLIE of crude streptococcal extracts into agarose-containing anti-E3 serum. Reference antigen was E3 crude extract (100 μg of carbohydrate per ml). Anode at top. (a) Crude extracts of *S. uberis* strains: 1, E3; 2, R1; 3, C1; 4, E2; 5, ATCC 19436. (b) Crude extracts of group B and E streptococcal strains: 1, group B untypable; 2, group B type Ia; 3, group B type Ib; 4, TG9 (group E); 5, ATCC 21223 (group E).

and standardized to 500 μg of carbohydrate per ml, using the indole test as described by Williams and Chase (23); D-glucose was used as a standard.

Purification of crude antigen. Antigens present within the crude extract were separated by gel filtration chromatography. A column (2.6 by 67.9 cm) of Sephacryl S-200 superfine gel filtration matrix (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was equilibrated with the extraction buffer, with the addition of 0.2% sodium azide and the deletion of the disodium EDTA. Fractions (7.5 ml) were monitored via a Uvicord III UV monitor (LKB, Rockville, Md.) and collected. Fractions were also monitored for carbohydrate content by the indole test.

Antisera. Production of antisera to the streptococcal strains was accomplished with the preparation of Formalin-killed bacterins, basically following the procedure of Lancefield (11). Flemish giant rabbits were inoculated three times weekly with 0.5 ml of the bacterin for the first week and with 1.0 ml in subsequent weeks until the serum was deemed adequate.

Immune serum against partially purified streptococcal extract was produced by first dialyzing the extract against 0.85% saline overnight to remove excess sodium azide and then emulsifying in a ratio of 1:1 with Freund incomplete adjuvant (Difco). A total of 2.0 ml of this emulsion was inoculated intramuscularly into each hind leg of a rabbit. The animal was test bled weekly until the serum was deemed adequate.

Anti-lipoteichoic acid serum as well as lipoteichoic acid from group A streptococci were supplied through the generosity of I. van de Rijn, Rockefeller University, New York, N.Y.

Immunodiffusion. A micromethod of the Ouchterlony technique (4) for precipitin reactions in agar gel, using a Plexiglas template on a glass microslide, was used. Noble agar (Difco) was made to 1% in 0.02 M phosphate buffer (pH 7.4) with the addition of polyethylene glycol (molecular weight, 6,000) to a final concentration of 3%, the latter modification according to Harrington et al. (8). Immunodiffusion slides were allowed to develop for 48 h in a moist chamber at room temperature and stained as described below.

Immunoelectrophoresis. The same buffer was used for all electrophoreses performed in this study. The stock buffer was prepared according to Weeke (21) and composed of 0.187 M Tris–0.374 M glycine–0.00112 M barbital–0.315 M sodium barbital (μ = 0.08; pH 8.8). The stock buffer was diluted 1:2 for use as the electrode buffer (μ = 0.04; pH 8.8) and 1:4 for use in the preparation of the 1% agarose (GIBCO Laboratories, Grand Island, N.Y.) for electrophoresis (μ = 0.02; pH 8.8). Electrophoresis was carried out in a Gelman electrophoresis chamber (Gelman Sciences, Inc., Ann Arbor, Mich.) equipped with a Plexiglas cooling plate connected to a circulating cold-water bath (PK pump; Fisher Scientific Co., Pittsburgh, Pa.).

Fused rocket immunoelectrophoresis (FRIE) was performed according to the method of Svendsen (19). The first 3 cm of a 10- by 10-cm glass plate was flooded with 4.5 ml of the agarose, and the remainder was flooded with 9.5 ml of agarose plus 1.0 ml of the appropriate antiserum. Gel filtration chromatography fraction samples (7.5 μl) were dispensed in 2-mm-diameter wells and allowed to diffuse for 30 min before electrophoresis into the antibody-containing gel at 2 mA per plate for 16 to 18 h at 4°C.

Rocket line immunoelectrophoresis (RLIE) was performed according to the method of Kroll (10). A reference antigen gel strip (5 by 90 mm) was cut from a gel (33 by 100 by 1.5 mm) cast by pouring 4.5 ml of 1% agarose with the addition of 0.5 ml of the appropriate antigen. The gel strip was positioned on a glass plate (10 by 10 cm) parallel to a barrier placed 30 mm from one end of the plate, and the strip was surrounded by 4.5 ml of agarose (contact gel). Agarose (9.5 ml) containing appropriate antiserum (1.0 ml) was poured on the remainder of the plate, and sample wells (4-mm diameter) were cut in the contact gel approximately 2 mm from the cathode side of the reference gel strip. Samples (20 μl) were electrophoresed into the antibody-containing gel at 2 mA per plate for 16 to 18 h at 4°C.

Immunoelectrophoresis plates were pressed, washed, and dried according to the method of Weeke (21) and stained in Coomassie blue (Bio-Rad Laboratories, Richmond, Calif.) and crocein scarlet (Bio-Rad) as described by Crowle and Cline (5).

Enzymatic treatment of antigen. Bovine pancreatic trypsin (type III; Sigma Chemical Co., St. Louis, Mo.) was used to digest the protein component(s) of the partially purified streptococcal antigen, following the method of van de Rijn et al. (20). The antigen-containing protein was subjected to digestion by trypsin by adding 0.5 ml of the enzyme (10,000 U per ml in 0.05 M

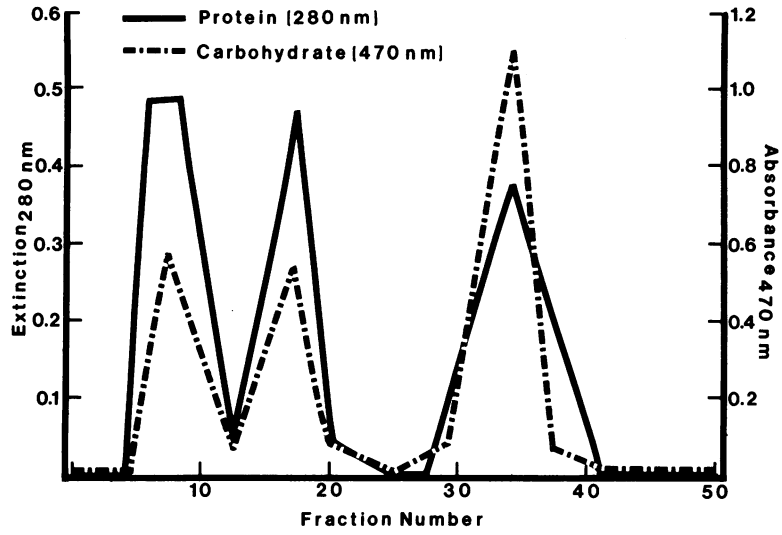

FIG. 2. Elution profile of E3 crude extract eluted through a Sephacryl S-200 SF gel filtration column (2.6 by 67.9 cm) with 0.05 M sodium phosphate-buffered saline, pH 7.4.

phosphate-buffered saline, pH 7.4) to 0.5 ml of the antigen and incubating at 37°C for 1 h. The reaction was then stopped by the addition of 0.5 ml of soybean trypsin inhibitor (Sigma) at a concentration of 5 mg per ml in the phosphate buffer.

RESULTS

Initially, several rabbits were inoculated with the Formalin-killed cells, using a random sampling of the *S. uberis* strains. Of the sera that were collected, only the serum produced against strain E3 contained precipitating antibody against all of the other strains. For this reason, strain E3 and its homologous serum, anti-E3, or derivatives thereof, were used exclusively as the reagents with and against which all other strains were tested.

RLIE of streptococcal crude extracts versus E3 and anti-E3. To show identity among the *S. uberis* isolates, a relatively sensitive immunoelectrophoretic technique, RLIE, was used. Figure 1 shows two plates in which strain E3 crude extract served as the reference antigen and anti-E3 served as the reference serum and on which samples of *S. uberis* and group B and E streptococcal crude extracts were electrophoresed. Reference antigen (Fig. 1a, well 1) precipitated four to five lines with the homologous serum, and all strains tested showed identity with at least the top two precipitin lines, including the group B (Fig. 1b, wells 1 to 3) and E (Fig. 1b, wells 4 and 5) strains.

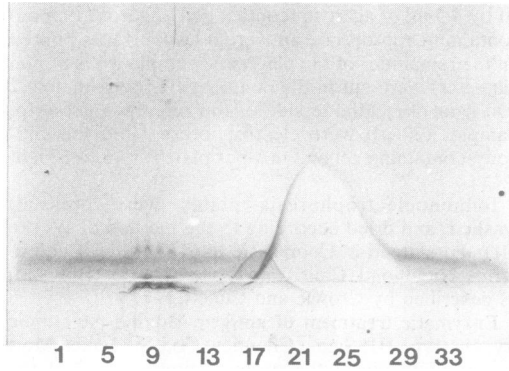

FIG. 3. FRIE of Sephacryl S-200 SF fraction samples into agarose-containing anti-E3 serum. Fractions were dispensed sequentially from left to right. Anode at top.

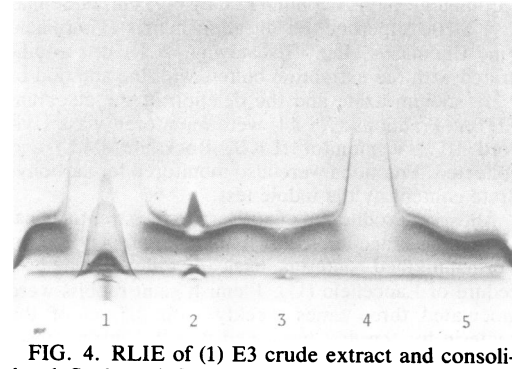

FIG. 4. RLIE of (1) E3 crude extract and consolidated Sephacryl S-200 SF fractions, (2) E3#1, (3) E3#2, (4) E3#3, and (5) E3#4 into agarose-containing anti-E3 serum. Consolidated fractions were concentrated to the original volume of sample applied to the column (5 ml), which had a carbohydrate concentration of 2 mg/ml. Reference antigen was E3 crude extract (200 μg of carbohydrate per ml). Anode at top.

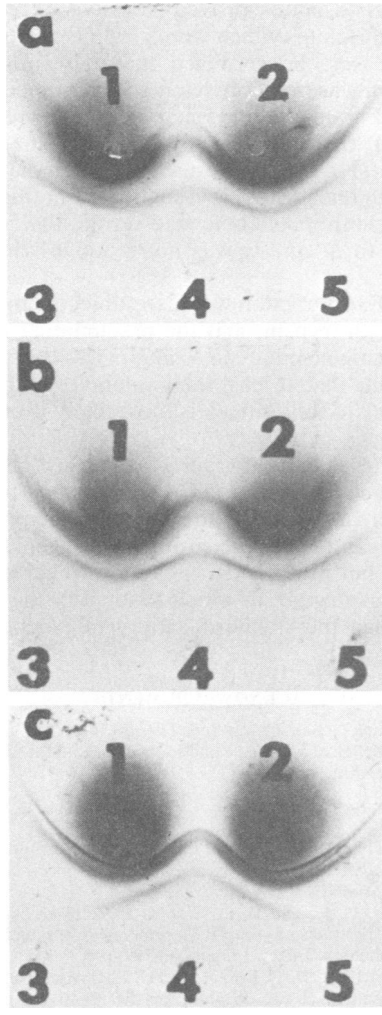

FIG. 5. Immunodiffusion of crude streptococcal extracts and E3#3 versus anti-E3#3 serum. Antiserum to E3#3 fills wells 1 and 2 in (a) to (c) and the homologous antigen(s) E3#3 fills well 4. Crude buffer extracts of several *S. uberis* isolates fill wells 3 and 5 of (a) and (b), and similar extracts of group E and B are present in wells 3 and 5, respectively, of (c).

Partial purification of E3 crude extract. After examination of the RLIE plates, it seemed desirable to attempt to purify the antigen(s) responsible for the identity reactions apparent on these plates. To accomplish this, crude extract was separated by Sephacryl S-200 SF gel chromatography. Fractions were monitored for both protein and carbohydrate content, and the resultant elution profile is presented in Fig. 2. Three main peaks resulted, each of which contained both protein and carbohydrate. Fractions were further analyzed by FRIE against anti-E3 serum (Fig. 3) and resulted in three main precipitin areas. The first area corresponded to the first elution peak and was designated E3#1. The second precipitin area contained two precipitin peaks, corresponded to the second elution peak, and was designated E3#2. The third FRIE precipitin peak also came from the second elution peak and was designated E3#3. The third elution peak gave no corresponding precipitin reaction on the FRIE plate, but was designated E3#4 nevertheless.

The fractions responsible for the precipitin peaks on FRIE were consolidated and concentrated, avoiding overlap of precipitin peaks as much as possible. These consolidated fractions were then electrophoresed versus anti-E3 serum with E3 crude extract reference antigen on RLIE. Figure 4 illustrates that fraction E3#3 (well 4) deflects both of the top two reference precipitin lines and that fraction E3#1 (well 2) deflects the top line and also forms two to three small precipitin arcs below the reference lines. E3#2 (well 3) and E3#4 (well 5) show no reference line deflections, although E3#2 does form a small precipitin arc.

Production and analysis of anti-E3#3 serum and fraction E3#3. From the results of the RLIE in Fig. 4, it was apparent that fraction E3#3 contained the antigen(s) responsible for the identity reactions among the crude extracts of streptococci tested. Figure 5 shows the results of immunodiffusion tests, using a serum produced against antigen E3#3 (anti-E3#3 serum) to compare crude extracts of *S. uberis* and group B and E streptococcal crude extracts for the presence of the common antigen(s). All of the *S. uberis* crude extracts (Fig. 5a and b) reacted with identity to the precipitin lines formed by the homologous system, E3#3/anti-E3#3. Group B and E extracts (Fig. 5c), however, only reacted with the two lines closest to the antibody well.

Trypsinization of E3#3. To determine whether the antigen specific for the *S. uberis* extracts contained any protein, semipurified E3#3 was subjected to treatment with trypsin. Immunodiffusion of the resulting solution (Fig. 6) showed

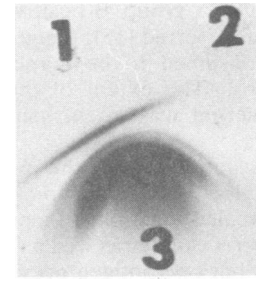

FIG. 6. Immunodiffusion of anti-E3#3 serum (well 3) versus intact (well 1) and trypsinized (well 2) E3#3.

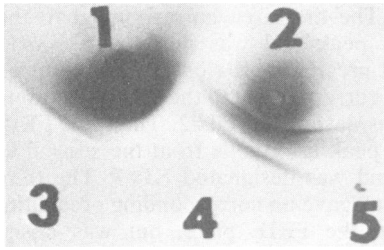

FIG. 7. Immunodiffusion of E3#3 (well 4) and lipoteichoic acid (wells 3 and 5) versus antilipoteichoic acid serum (well 1) and anti-E3#3 serum (well 2).

that the antigen specific for *S. uberis* was no longer present, although the other precipitin lines remained.

Assay for presence of teichoic acid. Since most, if not all, gram-positive organisms contain teichoic acid (9), it seemed prudent to determine whether the antigen unique to *S. uberis* was teichoic acid or contained teichoic acid. Figure 7 illustrates the immunodiffusion reaction of lipoteichoic acid (wells 3 and 5) and anti-lipoteichoic acid (well 1), E3#3 (well 4), and anti-E3#3 (well 2); it is apparent that E3#3 contains some component which precipitates the anti-lipoteichoic acid serum, but the antigen specific for the *S. uberis* isolates did not.

DISCUSSION

Initially, this study provided a serum, anti-E3, which would react with all of the *S. uberis* strains used. A relatively sensitive technique, RLIE, was used to detect this reaction of identity and indicated that at least two antigens were common among these isolates. Wessman and Shuman (22) produced a similar serum, detected by passive hemagglutination as a test assay, but only a total of 8 *S. uberis* strains were used as opposed to 53 in this study. The group B and E streptococci used in this study also showed reactions of identity in this system. Group E strains were originally used here because of the approximately 20% group E-positive population in *S. uberis*, so cross-reactions within this group were not totally unexpected. Since only one previous case of a group B-positive *S. uberis* strain had been reported (15), the group B organisms were presumed to be a relatively safe negative control. The extent of their reaction with anti-E3 serum was, then, somewhat surprising.

Immunodiffusion assays of the partially purified antigen, E3#3, and its homologous serum, anti-E3#3, revealed reactions of identity among all of the *S. uberis* isolates as well as the group B and E strains, but one antigen present in E3#3 was unique to the *S. uberis* isolates. Cullen's (6) attempt at isolating a common antigen among his *S. uberis* isolates proved negative except for those of isolates which were group E positive. In these cases, he surmised that the antigen in question was probably teichoic acid. Since probably all gram-positive bacteria contain teichoic acid (9), the present study examined the E3#3 antigen(s) for the presence of teichoic acid; although teichoic acid was detected in this fraction by anti-lipoteichoic acid serum, the antigen unique to *S. uberis* was not reactive with this serum.

The E3#3 fraction was also subjected to treatment with trypsin, and the precipitating ability of the antigen unique to *S. uberis* was destroyed, indicating that at least the immunologically specific area of this antigen is most likely protein in nature.

Further chromatographic or electrophoretic separation and biochemical analysis are indicated before a definitive label can be placed on this antigen as a possible grouping antigen for *S. uberis*, but it may provide a more reliable, less laborious manner in which to identify this organism than the standard battery of biochemical tests.

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